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14. ABSTRACT Fatty acid synthase (FASN), the enzyme that synthesizes fatty acid in cells, is overexpressed in prostate cancer and a potential therapeutic target. We have identified several novel chemical scaffolds with potential to inhibit FASN. An extensive series of anti-FASN pharmacophores has been synthesized and characterized for their ability to inhibit recombinant FASN, FASN activity in tumor cells, and to kill prostate cancer cell lines. The best inhibitors have increased potency over other FASN inhibitors, including orlistat, the prototype FASN thioesterase inhibitor. The current studies represent a significant advancement of the development of FASN inhibitors and represent an advancement of the translation FASN inhibitors into the clinic.					
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Introduction

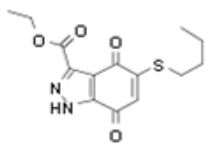
The purpose of this LCT award is to optimize chemical scaffolds as potential inhibitors of the thioesterase (TE) domain of fatty acid synthase (FASN). It is founded on the robust body of literature demonstrating that FASN represents a valuable drug target. Using an iterative scheme of *in silico* design, activity-based screening and structural analyses we identified a series of novel pharmacophores with inhibitory activity against FASN. This proposal has three specific aims. They are 1) To optimize FASN inhibitors through structure-based design, chemical syntheses and *in vitro* testing, 2) To determine the toxicological and pharmacokinetic properties of novel inhibitors, and 3) To test the efficacy of FASN inhibitors in human prostate cancer xenografts. Significant progress toward the development of novel and effective FASN inhibitors has been made. The progress from our team is outlined below.

Body

In this second project year focus was placed on Lead Series development based on outcomes from the first project year and on new scaffolds. Since the beginning of DOD-LCT funding our molecular design and medicinal chemistry efforts have led to the synthesis of more than 87 fully characterized compounds representing 13 structural classes. During project year two, **17** new compounds were characterized we are in the process of developing **6** additional structural classes: benzimidazole-4,7-diones, benzo[d]oxazole-4,7-diones, quinoxaline-5,8-diones, quinoline-5,8-diones, benzo-1,2,4-triazine-6(4*H*)-ones, 1*H*-indazole-4,7-diones (new series), pantothenate-linked 1*H*-indazole-4,7-diones and 1,2,3-triazoles. The novel members of the 1*H*-indazole-4,7-diones are the subject matter of one new provisional patent application. All salient data collected, in project year 2 is summarized in **Appendix A**.

In the course of further advancing the Lead Candidate discussed in last year's report (TPI-421) we determined that it has a half-life in solution of ~1 week, making it a less than ideal candidate for development. As outlined in **Section III**, we are aggressively pursuing a strategy to preserve the desired pharmacology of TPI-421 whilst increasing the overall chemical stability of this novel molecule. Because of its enhanced chemical stability of TPI-403, we are now advancing it for *in vivo* assessment against prostate cancer [discussed in **Section I**]. We report below on our progress to increase the overall solubility and pharmacological potency of the 1*H*-indazole-4,7-diones [**Section II**].

TABLE 1. *in vitro* data for TPI-403

chemical structure	
	
TE1 EC ₅₀	3.9uM
% inhibition of ¹⁴ C-acetate incorporation at 10uM	97%
MTS assay PC-3 EC ₅₀	3.3uM
crystal violet assay PC-3 EC ₅₀	5.2uM
MTS assay MCF-7 EC ₅₀	5.7uM
MTS assay FS-4 EC ₅₀	9.2uM
therapeutic index FS-4/PC-3	2.8

In addition, to help augment our discovery effort, during the second year of our DOD-LCT funded research we initiated another multilateral strategy: The development of other scaffolds similar to the 1,4-benzoquinone and 1*H*-indazole-4,7-diones; (b) the use of *in situ* click chemistry to develop novel scaffolds that make use of the chemical information contained in our current body of SAR data. Each element of the strategy- as well as our progress against objectives- will be discussed in **Sections IV** and **V**, respectively.

I. Supporting data and strategy for animal studies on TPI-403

Table 1 summarizes data for TPI-403, our current candidate for *in vivo* assessment and further optimization. Our team has engaged a Clinical Development Specialist and a Pathologist, both highly experienced in conducting pre-IND safety assessments. We are developing the protocols a robust determination of maximum tolerated dose (MTD) in mice. Body

weight and temperature and feeding will be monitored. Post mortem evaluation of organ weight and histopathology will be conducted to determine acute effects of this compound. If it is determined that TPI-403 possesses an acceptable MTD, we will evaluate this compound in a prostate tumor xenograft mouse model. Scale-up synthesis of TPI-403 is underway for the MTD studies and xenograft tumor model studies.

II. Update: Compound Optimization

As indicated in our Year 1 progress report, optimization of the indizoles (e.g., TPI-403, TPI-417, TPI-421) is focused on two themes: (1) increasing affinity at TE1 and (2) increasing solubility in aqueous media. The former goal can lead to an increase in therapeutic index (defined here as $EC_{50}[\text{normal cells}]/EC_{50}[\text{cancer cells}]$). Our structure-activity data show that a wide variety of substituents are accommodated in Regions A and B of the 1*H*-indazole-4,7-diones scaffold. These regions are depicted in Figure 1. For reference, structure-activity data for TPI-403, TPI-417 and TPI-421 are provided in Appendix A.

In Region A we are developing six new ring systems; discussed in detail in Section IV below. We believe ring systems will be synthetically more facile and One of the key challenges with the synthesis of 5-thio 1*H*-indazole-4,7-diones is that the indazole ring has to be introduced in the final synthetic step. We our synthesis and utilization of unsubstituted 1*H*-indazole- a synthetic intermediate have been unsuccessful. When combines 1,4-benzoquinone and diazomethane the strongly tends toward the addition of 2 diazomethane and the target compound itself suffers significant instability.

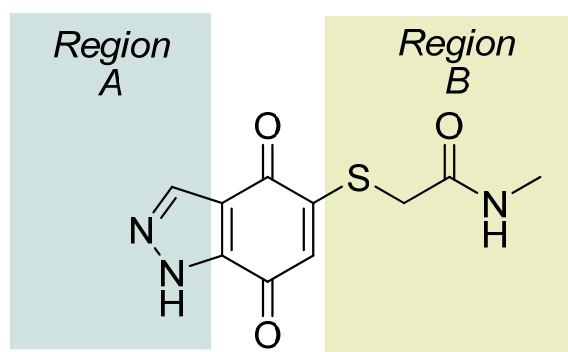


Figure 1- 1*H*-indazole-4,7-dione optimization regions

these will be that these more stable. substituted (region A) attempts at 4,7-dione as one reaction molecules chemical

Amino acid substituted indazoles. TPI-636 was synthesized with the intention of increasing solubility of the indazoles by introducing an amino acid sidechain into Region B, the intermediate itself shows potent inhibition of recombinant TE1, but this compound did not kill cancer cells. The FDA approved compound Orlistat, which was determined by our group to potently and irreversibly inhibit TE1 contains an N-formylated amino acid sidechain. Because of this and other factors we believe this as a promising direction for optimization that warrants more investigation.

Analogs containing pantetheine-mimetics. As reported last year, crystallographic and docking data generated by our laboratories provide information about the orientation of the 1,4-quinone moiety in the TE1 active site. We note that this binding mode puts the moiety in close proximity to the pantetheine channel of the enzyme. Together these data demonstrate that substituting a pantetheine moiety onto the 1*H*-indazole-4,7-diones position of the 1*H*-indazole-4,7-dione scaffold would preserve the likely binding mode of the quinone near the catalytic triad of TE1 while packing the pantetheine channel, which is a unique feature of TE1.

We surmised that the introduction of a pantetheine moiety in a favorable orientation will not only significantly increase TE affinity and solubility, but will also increase specificity of the series toward the target. Why? Because pantetheine is a cofactor used exclusively for fatty acid synthesis, which is an absolute requirement of epithelial cancer cells and is also known to correlate with tumor aggressiveness. For the sake of synthetic simplicity we initiated these efforts working with a truncated pantetheine sidechain that was synthesized from a novel ring-opening (D)-(-)-pantolactone using mercaptoethylamine, as shown in **Figure 2**, Panel A. Whilst we successfully synthesized the key intermediates 2i and 2iii we were unable to successfully synthesize the 1*H*-indazole-4,7-dione derivative 2iv. Interestingly, the benzoquinone compounds TPI-637 and TPI-638 potently inhibit recombinant TE1, yet show no effect on cancer cells or normal cells. In **Section IV** we show how the key intermediate 2ii is being used in other optimization efforts.

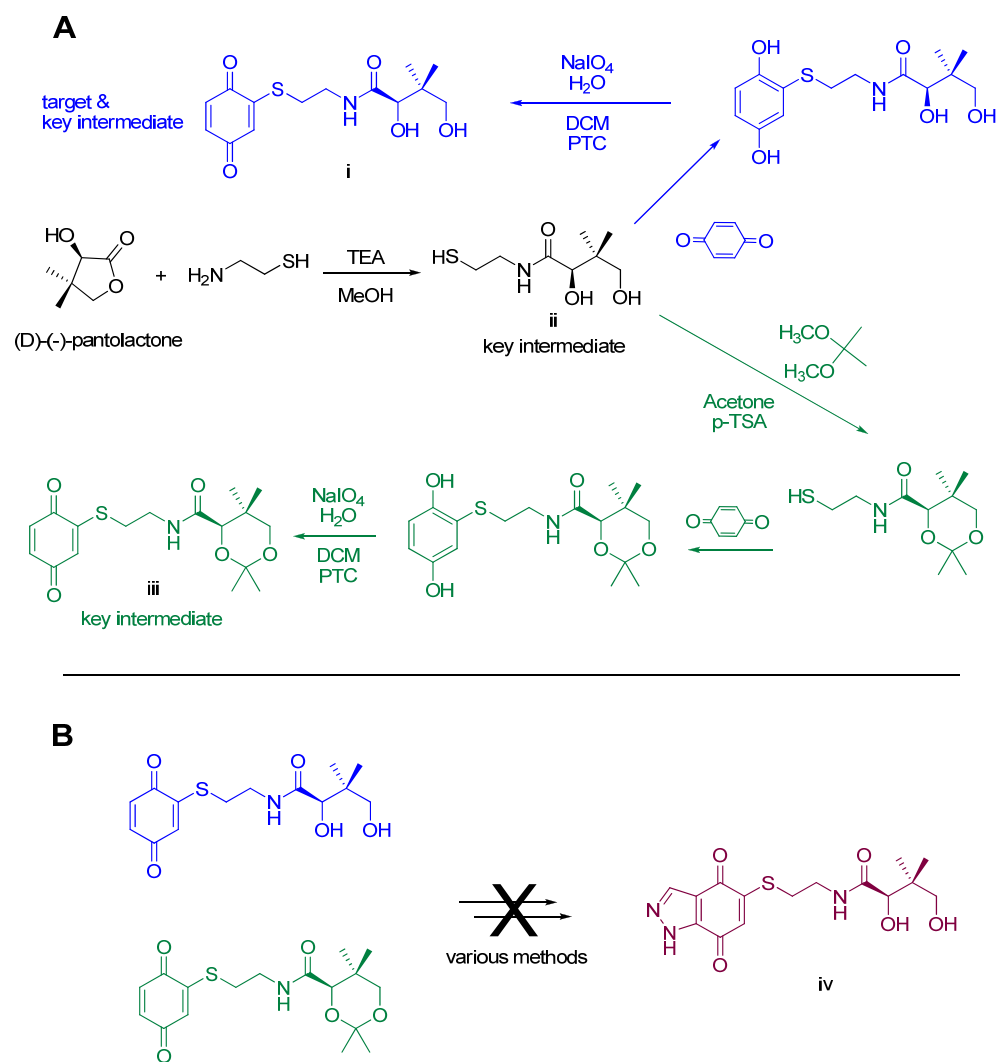


Figure 2. Synthetic strategy of truncated pantetheine analogs

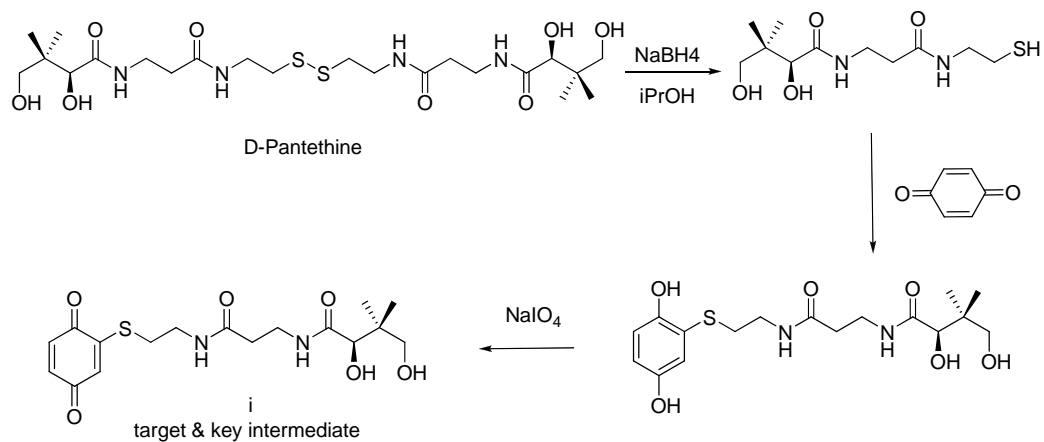


Figure 3. Synthetic strategy of pantetheine analogs

The initial strategy we have used to synthesize analogs with the full pantetheine sidechain is shown in **Figure 3**. These efforts have been hampered by challenges in purifying the key intermediate 3i. Evaluation of 3i (HHO3-79-I) for its ability to inhibit recombinant TE1 indicate that it is less potent than the equivalent truncated analog (TPI-638); further investigation will be required to determine the extent to which lack of overall purity might underlie this observation. Said lack of purity has also precluded definitive chemical characterization.

III. Update: increasing the chemical stability of TPI-421

Our studies suggest that the 1-position nitrogen of the 1*H*-indazole-4,7-dione is the source of chemical instability due to its high level of reactivity (**Figure 4**). It appears that loss of parent compound arises from two mechanisms: (1) ring opening, likely due to bond fissure between the two nitrogens (positions 1 and 2); (2) intermolecular attack by one TPI-421 molecule on a second. To increase stability, we are synthesizing the 1-N-methyl and 1-N-acetyl derivatives of the key synthetic intermediate, 4i and 4ii respectively. derivatives of TPI-421 as shown in **Figure 4**. In addition to a likely increase in chemical stability, acylation of the 1-N provides an additional position on the core molecule for optimization. The bottom scheme shows the synthetic route we are using to synthesize the N-1-substituted analogs of TPI-421 as well as other analogs, as the key intermediate will be used to synthesize a variety of analogs.

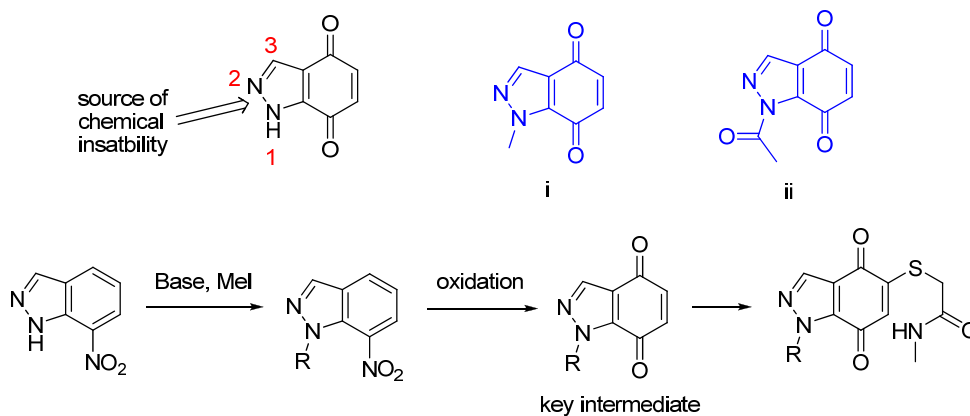


Figure 4. Strategies to increase the chemical stability of TPI-42

BACKUP COMPOUNDS AND OTHER FINDINGS

IV. The development of other scaffolds similar to the 1,4-benzoquinone and 1*H*-indazole-4,7-diones

In Region A (**Figure 1**) we are in the process of developing six new ring systems (**Figure 5**, structures i–vi). Thus far we have made thio-substituted analogs of quinoline-5,8-diones (2i) and benzo[*d*]oxazole-4,7-diones (2ii). Both of these classes show promising pharmacology (e.g., TPI-428, TPI-429), increased solubility, but will require more optimization. In the course of working with 2i it was observed that the reaction can be driven to form the 6,7-disubstituted analogs of quinoline-5,8-dione (e.g., TPI-429, TPI-435)- leading to compounds that should be more stable and more flexible with regards to introduction of moieties to increase solubility. These 6,7-disubstituted quinoline-5,8-diones appear to have good pharmacological profiles.

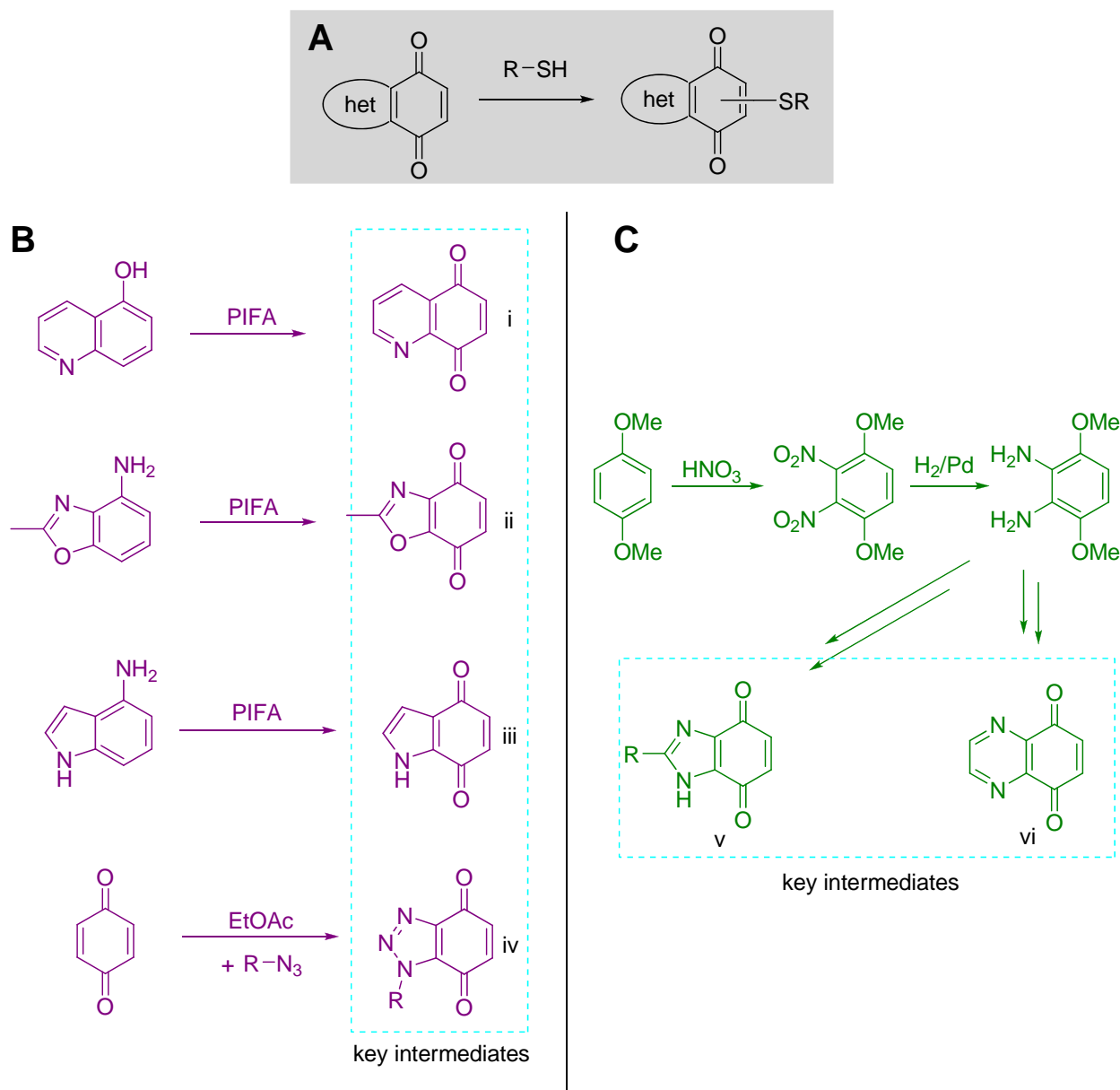


Figure 5. Outline and strategy to develop novel ring structures

Unlike the 1*H*-indazole-4,7-diones that require the indazole ring to be synthesized in the final step, these ring systems can be synthesized first and then chemically elaborated (**Figure 5**, panels A & B. As mentioned above, the synthesis of quinoline-5,8-diones (5i) and benzo[*d*]oxazole-4,7-diones (5ii) have been completed, the thio derivatives synthesized thus far have exhibited encouraging pharmacology. The 1*H*-indole-4,7-dione core (5iii) will be synthesized last, since the intellectual property around this class of structures is more constrained than the other 5 cores. Preliminary investigation of the 1*H*-benzo[*d*][1,2,3]triazole-4,7-dione scaffold (5iv) indicates that it can be synthesized easily in high yield.

The synthesis of 1*H*-benzo[*d*]midazole-4,7-diones (5v) and quinoxaline-5,8-dione (5vi) has been completed and purification is in process (**Figure 5**, panel C). We intend to aggressively move forward with parallel synthesis and pharmacological evaluation of mono- and di-substituted thio analogs of these new ring systems.

V. The use of *in situ* click chemistry to develop novel FASN inhibitors

We are currently augmenting our medicinal chemistry efforts with target-guided synthesis. This methodology offers an attractive alternative to traditional lead optimization techniques, by making use of the protein target as a nanoscale reaction vessel—only the building blocks that fit into the confines of the protein binding site(s) can react to form new compounds. Our group is employing a recent extension of this methodology—known as *in situ* click Chemistry (CC)—which uses the bioorthogonal Huisgen cycloaddition reaction to identify novel high affinity ligands; these and other investigators have shown that very high-affinity compounds can be identified with relatively little effort. In the CC experiment, a set of alkynes and azides are combined with target protein in aqueous buffer under ambient conditions. Those alkynes and azides that bind with an orientation favorable to cycloaddition form new triazolyl compounds.

To start, we are leveraging the useful information embodied in structure-activity data from this project's high-throughput screening work and from other sources. In this embodiment of CC, hits from high-throughput screening and other known active compounds are 'deconstructed' into components that can easily be converted into CC fragments (azides and/or alkynes). We are currently in the process of conducting trial CC reactions to determine the feasibility of this approach.

Key Research Accomplishments:

- Synthesis and characterization of 17 novel FASN inhibitor scaffolds. (see Appendix A)
- Optimization of FASN inhibitors of novel chemotypes
- Development of new synthetic strategies and avenues to generate FASN inhibitors

Reportable Outcomes:

Manuscripts

1. DeFord-Watts, L.M., Mintz, A. and **Kridel, S.J.**, The Potential of ^{11}C -acetate PET for Monitoring the Fatty Acid Synthesis Pathway in Tumors (2010) *Current Pharmaceutical Biotechnology*, *In press*

Funding received, based on this award

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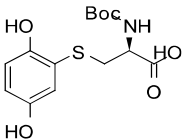
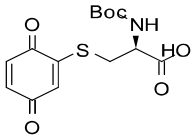
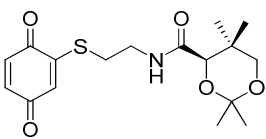
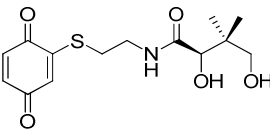
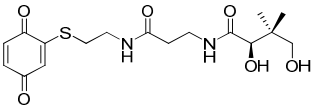
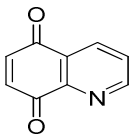
Fatty acid synthase inhibitors for cancer therapy: accelerating intellectual property creation through innovative chemistry

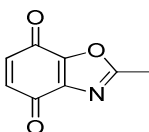
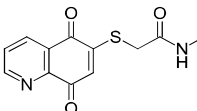
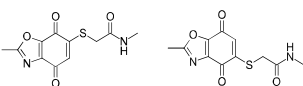
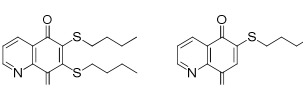
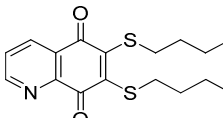
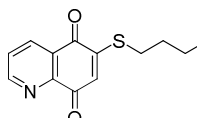
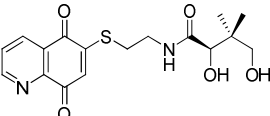
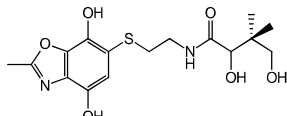
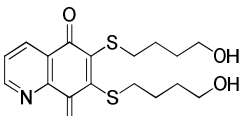
Conclusion

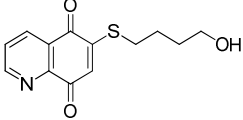
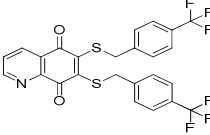
Our group has synthesized and fully characterized 87 novel FASN inhibitors that represent multiple pharmacophore classes. These results highlight the significant effort that has been put forth as well as the hurdles that have been overcome. The data also highlight the hurdles that remain and provide an illustration as to why FASN inhibitors have not yet been successfully translated to the clinic. Our success has been somewhat dampened by poor solubility and stability of several key compounds with promising attributes. Some inhibitors are able to inhibit recombinant enzyme, but are poorly soluble and unavailable to cellular FASN. Other compounds appear to have poor stability, limiting their potential as therapeutic agents. Despite these shortcomings, the wealth of knowledge that has been derived from these structure-activity relationships will be invaluable as this project moves forward. They will inform on the design of novel inhibitors as well as on the optimization of inhibitors that have demonstrated some promise against recombinant enzyme and in prostate tumor cells. In addition, we have concurrently initiated a click-based strategy for the enzymatic development of inhibitors by the FASN TE domain.

So what does this body of knowledge contribute? Several academic laboratories and pharma companies are developing inhibitors against FASN. The data from our findings will provide a foundation for inhibitors that target the

FASN-TE domain, in prostate cancer or other cancers. Novel scaffolds and potential binding modes have been identified. In addition, the incorporation of new synthetic strategies may also guide the development of FASN inhibitors or other therapeutic agents for cancer therapy. The work presented in this report highlight design and optimization of novel FASN inhibitors. This will contribute to the development of FAS inhibitors and provide an avenue toward the translation of FAS inhibitors into the clinic for potential use in treating men with prostate cancer.

Structure	TPI	recombinant thioesterase			% inhibition of	% relative survival (or IC ₅₀)			therapeutic
	Number	% Inhibition (10μM)		IC ₅₀ (μM)	¹⁴ C-acetate incorp	tumor cells		normal cells	index
		TE1	TE2	TE1	PC3 cells at 10uM	PC-3	DU-145	FS-4	FS-4/PC-3
reference compounds from project year 1									
	403-00-A								
	417-00-A								
	421-00-A								
project year 2 compounds									
	635-00-A	28	10	ND	ND	ND	ND	ND	ND
	636-00-A	81	100	ND	ND	100	ND	100	0
	637-00-A	91	94	2.49	0	100	ND	100	0
	638-00-A	100	96	1.48	0	100	ND	100	0
	HHO3-79-I only 70% purity	34	ND	ND	ND	ND	ND	ND	ND
	640-00-A	100	98	ND	ND	ND	ND	ND	ND

Structure	TPI Number	recombinant thioesterase			% inhibition of	% relative survival (or IC ₅₀)			therapeutic
		% Inhibition (10μM)		IC ₅₀ (μM)	¹⁴ C-acetate incorp	tumor cells		normal cells	index
		TE1	TE2	TE1	PC3 cells at 10uM	PC-3	DU-145	FS-4	FS-4/PC-3
	639-00-A	100	97	ND	ND	ND	ND	ND	ND
	427-00-A	96	97	0.76	IC ₅₀ =7.9uM	IC ₅₀ =6.9uM	IC ₅₀ =22uM	IC ₅₀ =3uM	0.4
	428-00-A	100	98	0.65	IC ₅₀ =9.1uM	IC ₅₀ =8.8uM	IC ₅₀ =18uM	IC ₅₀ =23uM	2.6
	429-00-A	100	100	0.72	in progress	IC ₅₀ =6.2uM	ND	IC ₅₀ =17uM	2.8
	429-00-B	100	100	in progress	in progress	in progress	in progress	in progress	in progress
	432-00-A	100	100	in progress	in progress	in progress	in progress	in progress	in progress
	430-00-A	86	100	4.72	ND	100	ND	100	1
	431-00-A	48	52	in progress	in progress	in progress	in progress	in progress	in progress
	433-00-A	96	99	in progress	in progress	in progress	in progress	in progress	in progress

Structure	TPI Number	recombinant thioesterase			% inhibition of ¹⁴ C-acetate incorp PC3 cells at 10uM	% relative survival (or IC ₅₀)			therapeutic index FS-4/PC-3
		% Inhibition (10μM)		IC ₅₀ (μM)		tumor cells		normal cells	
		TE1	TE2	TE1		PC-3	DU-145	FS-4	
	434-00-A	97	99	in progress	in progress	in progress	in progress	in progress	in progress
	435-00-A	100	100	in progress	in progress	in progress	in progress	in progress	in progress